

MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MLPA) ON-CHIP

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ABSTRACT

Polymerase chain reaction (PCR) has become an indispensable technique in clinical diagnostics. For integrated, lab-on-a-chip (LoC) solutions, multiplexed implementations of PCR are especially attractive; we describe the transfer and optimization of multiplex ligation-dependent probe amplification (MLPA) directly onto a silicon-based microfluidic chip. A standard, commercially-available MLPA protocol involves discrete steps: (1) probe hybridization, (2) ligation and (3) standard PCR amplification. Size separation can then be performed on the products to identify the different fragments. We describe the optimization of all MLPA reaction steps for use on-chip and show the fabrication of a single, integrated silicon fluidic chip for performing an entire MLPA protocol. These results describe a clear route towards a complete lab-on-a-chip system that integrates sample preparation, multiplex DNA amplification and detection onto a single disposable piece of silicon.

KEYWORDS: Multiplex Ligation-Dependent Probe Amplification (MLPA), PCR on-chip, silicon microfluidics

INTRODUCTION

Because the utility and high sensitivity of PCR, great efforts are being made to integrate the reaction in disposable chips or cartridges. Several research groups and companies are aiming to additionally include sample preparation in order to avoid cross-contamination while increasing reproducibility. Additional miniaturization and multiplexing will enable

further increases in throughput. To this end, we describe the use of multiplex ligation-dependent probe amplification (MLPA) on a silicon chip. MLPA, first described by Schouten *et al.* [1], allows amplification of up to 50 genomic fragments in a single reaction. Generally, MLPA consists of 3 discrete steps (see Figure 1). In a first step, DNA probes (which have a sequence complementary to the target of interest, a generic overhang and a stuffer sequence) will hybridize adjacent to each other. The second step, ligation, occurs only if both probes hybridize to the target. In a third step, using the generic overhangs, ligated probes will be amplified using a single, generic primer couple. As such, PCR biasing is avoided and the relative number of amplified fragments represent the relative amount of target sequences present in the sample. Additionally, because the ligase reaction is sensitive to single nucleotide polymorphisms (SNPs), multiple applications on-chip can be envisioned. For detection, the amplified fragments (each with a different length dependent on the stuffer sequence) can be size separated.

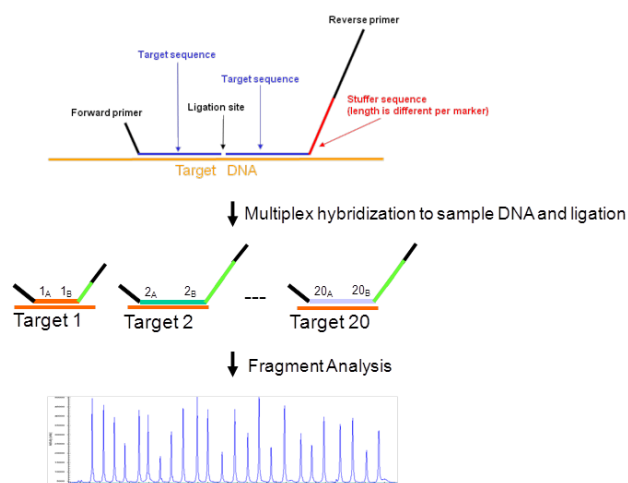


Figure 1: Schematic overview of the MLPA principle. Probes hybridize adjacent to each other and are ligated. Ligated probes are amplified using universal primers. The amplified fragments are separated by size for detection.

EXPERIMENTAL

For a proof-of-principle, the SALSA MLPA probe-mix A012 (MRC Holland, The Netherlands) was selected, which contains three probe couples that recognize different sequences of the human ERBB2 gene, one probe couple for the TOP2A gene, and eight reference probe couples binding to different autosomal chromosomal locations. For on-chip amplification, all MLPA reaction steps were reduced to 2 μ l volumes for compatibility with the designed and fabricated microsystem (see Figure 2). Furthermore, the hybridization step was reduced in time from the recommended (by MRC Holland) 16 h to 1 h. Before planned future work showing the implementation of the entire MLPA protocol in a single integrated microsystem, all MLPA sub-steps were optimized individually on-chip, meaning one step was performed on-chip while the other two steps were performed on a conventional PCR system. Results were always compared to the reaction performed on a conventional tool following the protocol as described by the supplier. For implementation of the reactions on-chip, the buffers and enzymes provided with the kit were used except for the final PCR step where various

enzymes were tested, namely the MLPA polymerase (supplied with the kit), KOD polymerase (Merck Millipore) and Titanium Taq polymerase (Clontech). Amplification of the different fragments using human genomic DNA as a template was visualized using a MultiNA microchip electrophoresis system (Shimadzu). Additionally, a chromatography filter based on a silicon micropillar array, as described previously [2], was tested to separate the MLPA amplicons using ion-pair reversed phase chromatography at 55 °C and a flow rate of 1 μ l/min. For separation, the acetonitrile concentration was linearly increased from 7 to 15% over 20 min. The separated fragments were observed using an external UV (254 nm) detector.

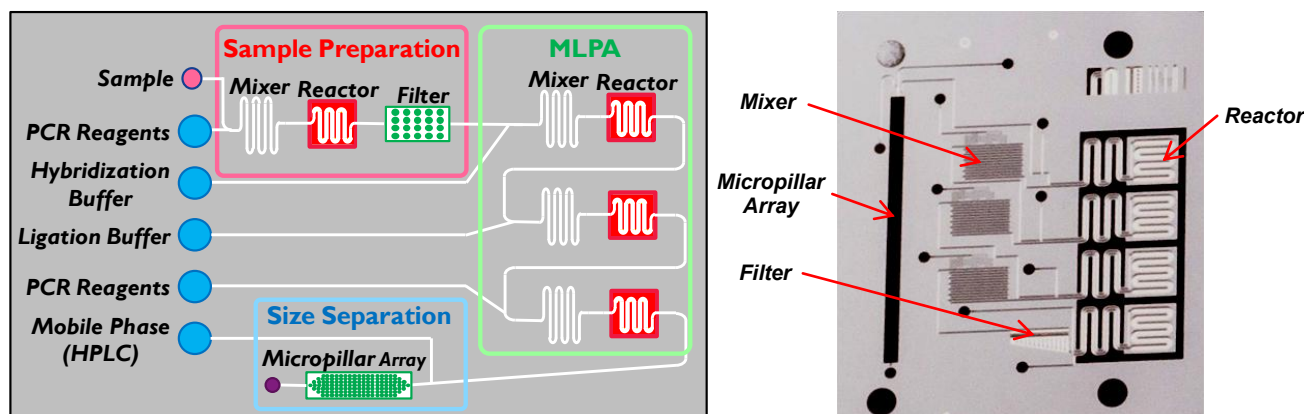


Figure 2: left: Schematic overview of the chip designed to enable MLPA (hybridization, ligation and PCR) and size separation of the amplified fragments using a micropillar array. Right: Picture of the prototype microsystem. The overall size of the test chip is 3.2 cm by 3.0 cm.

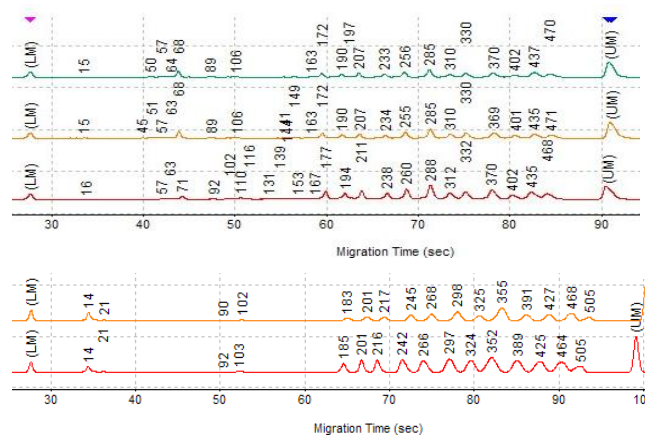


Figure 3: top: Separated MLPA fragments after performing all steps of the reaction using a conventional tool (red), performing ligation on-chip (orange), or performing the 1h hybridization step on chip (green). bottom: Separated MLPA fragments after performing the PCR step on-chip using Titanium Taq polymerase (orange) compared to using the standard protocol on a conventional PCR tool (red). The numbers indicate the size of the amplified fragments in base pairs.

SALSA polymerase (see Figure 3). The separation of the 12 successfully amplified MLPA fragments was also tested on chip using a micropillar filter. 7 out of 12 of the amplified fragments could be easily discerned from the chromatogram (see figure 4).

DISCUSSION

MLPA enables simultaneous amplification of a relatively large number of genetic DNA fragments in one single reaction. The protocol includes a ligation step that is nucleotide specific, hence, the technology has been used for the detection of single nucleotide polymorphisms (SNPs) as well as epigenetic markers [4]. The multiplexing capabilities of the technique are quite attractive for molecular diagnostics. However, the current MLPA protocol includes several manual handling steps. Implementation of the entire reaction on-chip would largely increase its ease-of-use. The current MLPA protocol is optimized as a single tube method, but still requires different mixing steps. For on-chip

RESULTS

The designed MLPA chip was fabricated successfully, integrating cavities for all MLPA reaction steps as well as a micropillar filter to separate the amplified fragments (see Figure 2). However, initial tests were conducted using single microreactor devices originally presented by Jones *et al.* [3]. All three MLPA sub-steps were individually performed on-chip using such devices. More particularly, and as shown in Figure 3, the hybridization and ligation step were successfully performed on-chip using the reagents supplied with the SALSA MLPA kit. The obtained peak pattern after separation was similar to the pattern obtained for an MLPA reaction done using a conventional PCR tool. In the commercial PCR tool, reducing the probe hybridization time from 16 to 1 hour did not appear to adversely impact the obtained peak pattern. For this reason, a 1 hour probe hybridization step was used for all subsequent experiments. For the on-chip micro-PCR step, no amplification could be observed when the SALSA polymerase supplied with the kit was used, while the KOD polymerase gave only very small peaks (data not shown). In contrast, the Titanium Taq polymerase resulted in clearly resolvable peaks similar to the MLPA protocol performed off-chip using the

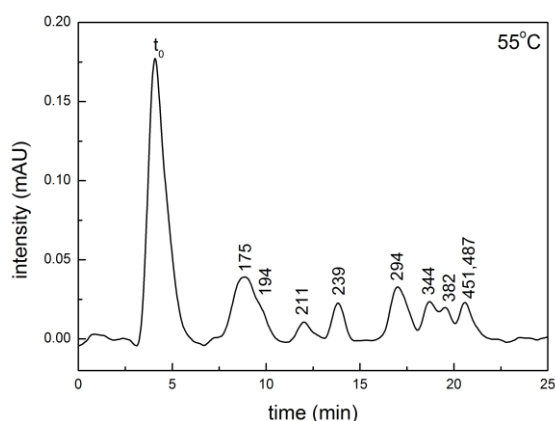


Figure 4: A size-based separation of the 12 MLPA fragments on-chip with a micro-pillar filter using ion-pair reversed phase chromatography at 55 °C and a flow rate of 1 μ l/min.

previously demonstrated in these silicon-based microreactors with surrounding air-gaps for thermal isolation [3]. Because of these high ramp rates, reductions in the PCR reaction time are likely obtainable. Another benefit is the possibility to include sample preparation and detection on the same microfluidic chip. Although we did not test MLPA directly from blood using the designed sample preparation module (see figure 3), we have shown successful DNA amplification on-chip directly from blood previously using a similar microsystem albeit with a different PCR assay [3]. Additionally, separation of the fragments based on size was tested on-chip as well. Using the conventional MLPA protocol, a capillary electrophoresis device is needed to discriminate the amplified fragments, which may not be available in every lab. Integrated on-chip size separation would largely simplify the overall protocol. Preliminary tests using a 2 cm long micropillar filter array (with 2 μ m pillar diameter and 1 μ m interpillar spacing) showed that already 7 out of 12 fragments could be clearly separated. Using the current filter design and an optimized protocol separation of all the peaks will be possible. The separation of up to 50 fragments, as often reported for MLPA, would likely require another new and more advanced filter design.

CONCLUSION

We show the fabrication of a disposable silicon microfluidic chip that couples sample preparation, microreactors and detection based on size-discrimination for a highly integrated solution for MLPA-based diagnostic testing. In short, although some further testing is needed to demonstrate the full MLPA on-chip assay, this work demonstrates that on-chip highly-multiplexed DNA detection is one step closer to the hands of life science researchers as well as clinicians.

ACKNOWLEDGEMENTS

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implementation, we have chosen to use separate cavities with a mixing unit for each individual step (i.e. probe hybridization, ligation and PCR) to largely simplify the microfluidic actuation. As described in the previous section, all individual steps were performed successfully on-chip. The only difference from the standard MLPA kit is the use of a different polymerase (Titanium Taq) in the microsystem. Possibly, the SALSA enzyme strongly binds to silicon walls as we have observed variable results in the past when applying different chemistries to coat the chip surface (data not shown). As avoiding extra coating steps is largely preferred, we have screened different polymerases. Based on these results, we are confident that running all reactions together on-chip will be successful as will be tested in the near future using the available MLPA chips (see figure 3). In addition to the automation, several other benefits are offered by the use of a miniaturized microsystem. Because of the small volumes used, rapid temperature ramp rates have been